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Hormones in wheat kernels during embryony

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Summary

Abscisic acid (ABA), indole-3-acetic acid (IAA) and six cytokinins in wheat (*Triticum aestivum* L.) grain components were extracted, from 0 to 25 d post anthesis (DPA), and quantified by noncompetitive indirect ELISA. At 3 DPA, which coincides with early embryo and endosperm cell divisions, whole kernel hormone levels in μmol kg^{-1} dry mass (DM) were: zeatin (Z) 30, IAA 4, zeatin riboside 1.8, ABA 1.0, other cytokinins < 0.6. Endosperm Z content declined from about 13 μmol kg^{-1} DM at 6 DPA to 0.1 μmol kg^{-1} DM by 9 DPA and remained low thereafter. Generally by 9 DPA, embryo differentiation is nearly complete, and the endosperm mitotic index has decreased to near zero. At 13 DPA, Z content in embryos was 0.9 μmol kg^{-1} DM, and then decreased to 0.3 μmol kg^{-1} DM by 25 DPA. Endosperm IAA content remained low through 9 DPA (about 6 μmol kg^{-1} DM) and then increased sharply to about 38 μmol kg^{-1} DM by 19 and 25 DPA. In contrast, embryo IAA content decreased from about 34 μmol kg^{-1} DM at 13 DPA to about 15 μmol kg^{-1} DM at 19 and 25 DPA. Endosperm ABA content fluctuated between 1.0 and 4.5 μmol kg^{-1} DM between 6 and 25 DPA while embryo ABA content fluctuated between 3.4 and 4.8 μmol kg^{-1} DM between 13 and 25 DPA. These hormone changes may be involved in the coordinated development of embryos in ovulo.

Key words: development – ELISA – embryology – plant growth regulators – seed fill

Abbreviations: ABA = abscisic acid. – diHZ = dihydrozeatin. – diH[9R]Z = dihydrozeatin riboside. – DPA = d post anthesis. – DM = dry mass. – FM = fresh mass. – IAA = indole-3-acetic acid. – iP = isopentenyl adenine. – [9R]iP = isopentenyl adenosine. – m-IAA = methylated indole-3-acetic acid. – MAb = monoclonal antibody. – Z = zeatin. – [9R]Z = zeatin riboside

Introduction

The angiosperm embryo develops in a chemical environment that initially is largely regulated by maternal tissues. Abnormal embryo development occurs when this environment is modified. For example, oblation of ABA synthesis in seeds following anthesis disrupts induction of embryo dormancy in wheat (Rasmussen et al. 1997), maize (Hole et al. 1989), and tomatoes (Groot and Karssen 1992). In maize, abnormal vivipary is prevented in ABA-deficient lines if ABA is supplied to kernels between 13 and 15 DPA (Hole et al. 1989).

The extent to which hormone and nutrient fluctuations direct normal embryo development in ovulo is unknown. This report is part of a larger project designed to characterize

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physical and biochemical factors of wheat ovules important in regulating embryony in ovulo and improving zygotic and somatic embryony *in vitro*. We previously described levels of water soluble carbohydrates, free amino acids, and elements in wheat kernals during early embryony (Carman et al. 1996) and showed how their simulation improved somatic embryogenesis *in vitro* (Carman 1995). In the present paper, we describe fluctuations in ABA, IAA and cytokinin levels, which were quantified for specific grain fractions during early embryony, rapid embryo growth, and the soft and hard-dough stages.

Materials and Methods

Plant material

Two populations of a hard red spring wheat line, PCYT-20, were grown in a greenhouse, at different seasons (fall and winter), using supplemental lighting and a soilless medium (Carman et al. 1988). Only the first and second spikes of healthy, vigorously-growing plants were used for analysis. From the first population, entire kernels were collected at 0 and 3 DPA, liquid endosperm was collected at 6 and 9 DPA (by rupturing individual kernels and collecting the fluid with a pipet), and embryos and endosperm were collected at 13, 19 and 25 DPA by physically separating them from other kernel tissues. From the second population, embryos were collected at 12, 18 and 24 DPA and whole kernels were collected at 0, 3, 6, 12, 18 and 24 DPA. These kernels were separated into a liquid and solid component by tissue disruption and centrifugation (Carman et al. 1996). Three independent samples were collected from each fraction of each population. All samples were immediately placed on ice, weighed to obtain FM, and frozen in liquid N_2. Frozen samples were lyophilized, and DM values were obtained.

Hormone extraction and purification

Lyophilized samples were homogenized in 80 % methanol (Brinkmann, Polytron) and spiked with DL-cis,trans-[G-^{3}H]abscisic acid (TRK.644, Amersham), 4.25 TBq $\times$ mmol^{-1} specific activity; 3-[5(n)-^{3}H]indolylacetic acid (TRK.781, Amersham), 1.04 TBq $\times$ mmol^{-1} specific activity; and ^{3}H-*trans*-[9R]Z, ^{3}H-diH[9R]Z and ^{3}H-[9R]iP, each of which were synthesized using nucleoside phosphorylase (Scott and Horgan 1980). Extracted hormones were purified by C_{18} octadecyl silica solid phase chromatography and HPLC (MacDonald and Morris 1985) using an ODS C_{18}, 250$\times$4.6 mm, 5 mm column (Alltech Associates). HPLC fractions (0.5 min) were collected, and a 20 µL aliquot was taken from each HPLC fraction for scintillation counting. Fractions containing ^{3}H-hormone were pooled, and fractions containing the cytokinins Z, diHZ and iP, which did not have corresponding ^{3}H-tracers, were located by reference to the respective cytokinin riboside ^{3}H-tracers (Fig. 1) and pooled. The purified hormone samples were lyophilized to dryness, and the IAA samples were methylated at the carboxyl site by esterification using ethereal diazomethane (Cohen 1984).

Non-competitive indirect ELISA

Endogenous hormones were quantified by a non-competitive indirect ELISA modified from Walker-Simmons (1987). The modifications allowed use of 96-well polypropylene assay plates for the initial sample/MAb incubation (Hess and Carman 1993, Banowetz et al. 1994). The hormone standards + ABA (Sigma), m-IAA (Aldrich) and cytokinins (*trans*-Z, *trans*-[9R]Z, diHZ, diH[9R]Z, iP and [9R]iP [Sigma]) were prepared by dissolving in a minimal amount of methanol and diluting to the standard concentrations with tris buffered saline. Tissue culture grade hormones were HPLC-purified prior to use as standards. The +ABA MAb was purchased from Idetek, Inc (# 8015). The m-IAA MAb was provided by John Caruso (University of Cincinnati, Cincinnati, OH) and prepared as in Leverone et al. (1991). The anti-cytokinin MAbs included anti-*trans*-[9R]Z# 3 (Trione et al. 1985), anti-dHZ[9]R# 4 (Banowetz et al. 1997), and anti-[9R]iP# 3 (Trione et al. 1987a). All wells of each 96-well plate were used. To minimize edge effects, materials used in the ELISA procedure were equilibrated to 25 °C.

Using an automated pipet station (Beckman Biomek 1000), the incubated sample-MAb mixtures were transferred to microplate (Immulon II 96-well flat-bottom, Dynatech Laboratories) wells coated with the respective hormone-protein conjugates. The ABA-C_4-BSA conjugate was prepared as in Walker-Simmons (1987). Cytokinin-BSA conjugates were prepared as in MacDonald and Morris (1985). The IAA-ovalbumin conjugate was prepared as in Weiler (1981) except IAA was used without recrystallization and ovalbumin (A-5503, chicken egg fraction V, Sigma) was used in place of BSA. All protein conjugates were stored and diluted in 0.05 mol/L bicarbonate buffer. Antibodies not complexed with sample hormone during the initial noncompetitive sample/MAb incubation (in polypropylene assay plates) were subsequently complexed to the conjugated hormone of the coated well surfaces of the Immulon II 96-well flat-bottom assay plates. Alkaline phosphatase-conjugated goat anti-mouse antibodies were then used to quantify the immobilized antibodies on the surfaces of the rinsed Immulon II 96-well flat-bottom plates. ELISA incubation periods, solution exchanges, and absorbency data collection were as in Walker-Simmons (1987) except that the ELISA solution volumes were reduced from 200 to 100 µL per well. A logit-log transformation was used to extrapolate hormone concentrations from absorbency readings (Weiler et al. 1981). Final hormone concentration measurements were corrected for losses based on recovery of ^{3}H-hormone immediately prior to conducting the ELISA (Walker-Simmons 1987).

Data analysis

Hormone concentration data were analyzed as completely randomized factorials (tissues and collection times) using the general linear model option of SAS (1987). Three replications were performed for all hormone and physical parameter (FM, DM) measurements. Four dilution aliquots (1 : 0, 1 : 1, 1 : 3 and 1 : 7) were taken for each tissue replication, and three replications of each aliquot were analyzed as repeated measures. Observations not falling within the respective standard curves were discarded. Differences between tissues, grain fractions, and DPA were tested using type III sums of squares, and the error terms were derived by nesting the tissue by replication interactions within DPA. These error terms were also used to derive standard errors for the least square means.

Results

Multiple hormone analyses from small samples

Our HPLC procedures separated +ABA, IAA, Z, [9R]Z, diHZ, diH[9R]Z, iP and [9R]iP from small samples (20 to 50 mg

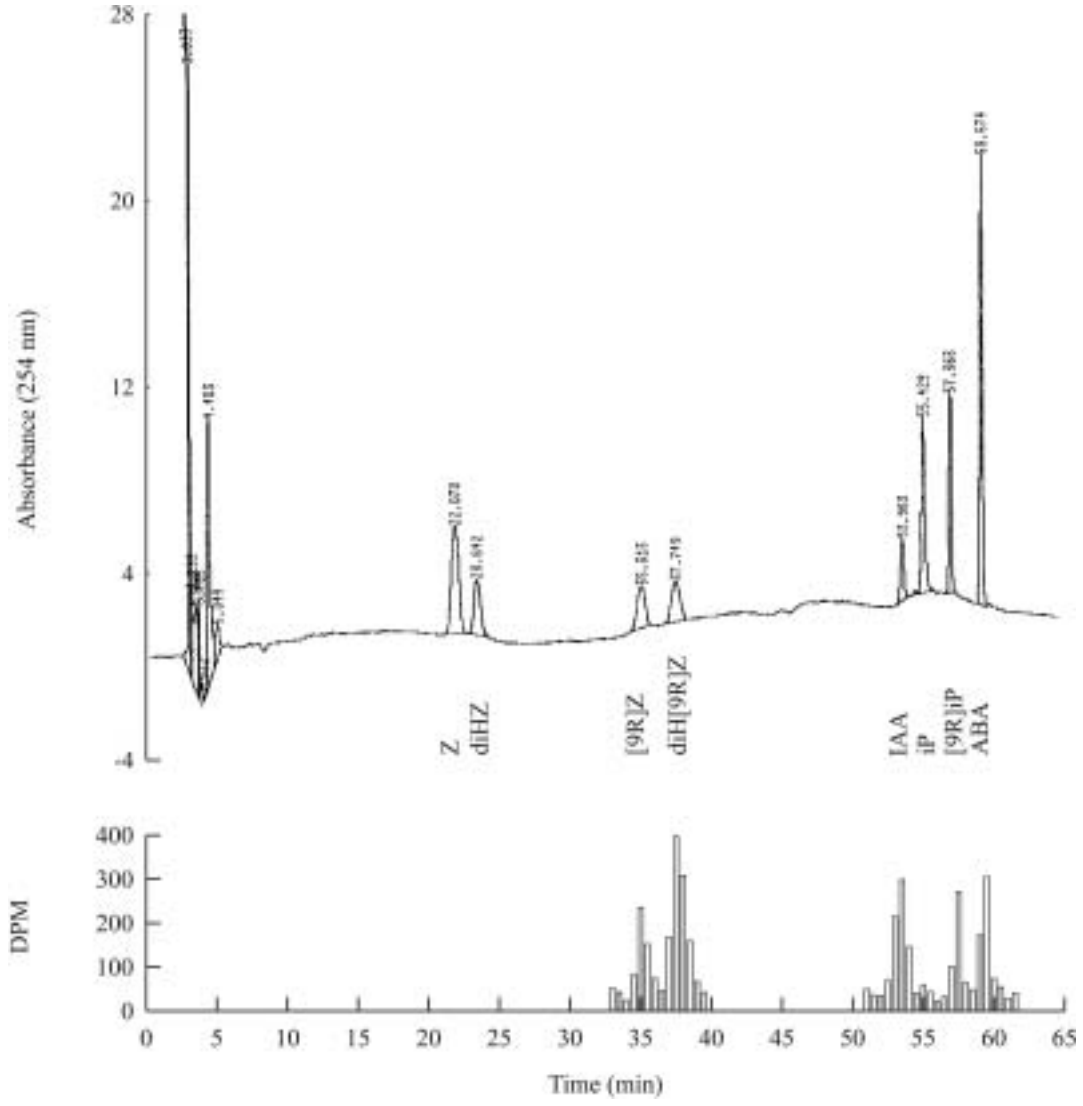

Figure 1. HPLC chromatogram of a hormone standard (50 ng of each hormone) and DPM counts from fractions of a purified tissue sample. Note that ^{3}H-hormone tracers correspond to chromatographic retention times of the respective hormones.

DM), and the ^{3}H-hormone tracers allowed hormone-containing HPLC fractions to be pooled (Fig. 1). Recovery percentages were: +ABA 31.3 ± 6.3 %, IAA 34.2 ± 6.8 %, Z and [9R]Z 33.2 ± 6.6 %, diHZ and diH[9R]Z 51.3 ± 9.6 %, and iP and [9R]iP 18.9 ± 3.8 %. The measurement range of the noncompetitive indirect ELISA depended on the MAb used (Fig. 2). The +ABA MAb was the most sensitive but had the narrowest range. The m-IAA MAb was the least sensitive but had the widest range. Cross reactivity of the latter MAb has not previously been reported, but our studies suggest that it is low as demonstrated by near parallel shifts in the m-IAA standard curves when HPLC-purified wheat grain extracts (approximately 40 mg L^{-1} of m-IAA at 1 : 0) were added to the standards (Fig. 3). The estimated amounts of m-IAA added and the amounts reported by the assay were also in agreement. Cross reactivities for the other MAbs used are also low (Trione et al. 1987 b, Banowetz et al. 1994, 1997, Hess and Carman 1998).

Grain growth

In both of the plant populations, grain moisture content increased up to 18 DPA but began to decline by 24 DPA (Fig. 4, compare FM and DM values), which is characteristic of the transition from the soft to the hard-dough stages. DM continued to accumulate through 24 DPA. The volume of the grain supernatant (population 2) increased up to 12 DPA and then decreased (Fig. 4). However, percentage DM in the grain supernatant fraction declined after 6 DPA, which correlated with

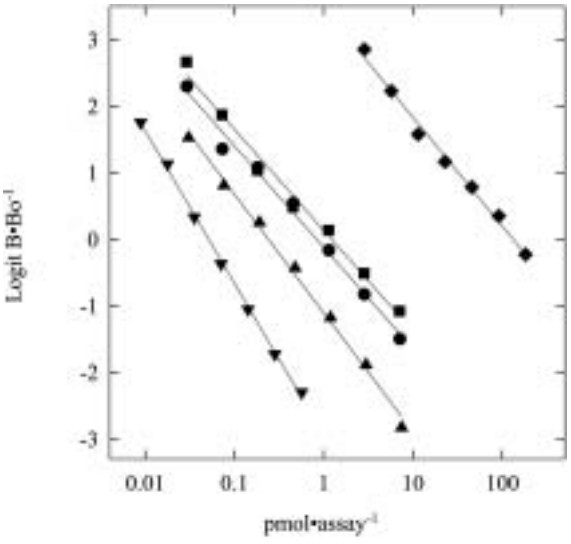

Figure 2. Logit-log-linearized standard curves for noncompetitive indirect ELISAs: +ABA, r^2 = 0.955; m-IAA, r^2 = 0.955; *trans*-[9R]Z, r^2 = 0.947; diH[9R]Z, r^2 = 0.959; [9R]iP, r^2 = 0.986. Each curve displays the effective measuring range of the respective MAb.

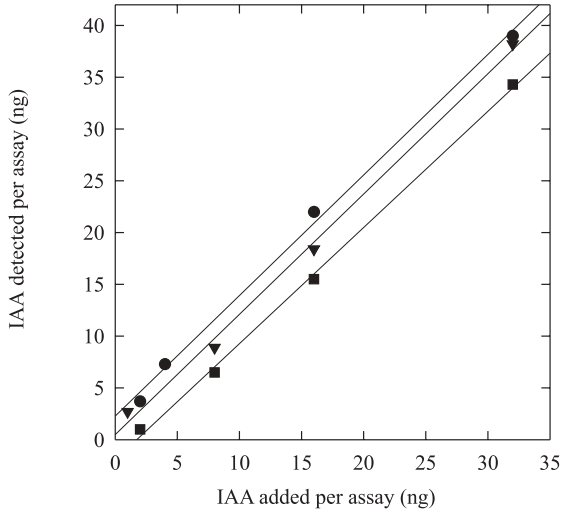

Figure 3. Dilution analysis of HPLC-purified IAA from wheat kernel extract: m-IAA standard (no extract), extract at a 1:10 dilution added to each m-IAA standard; extract at a 1:7 dilution added to each m-IAA standard.

a rise in osmotic potential (data not shown). Embryo DM in population 1 increased from 13 to 25 DPA (Fig. 4). In contrast, embryo DM in population 2 increased 5-fold from 12 to 18 DPA but did not increase thereafter. The ratio of FM to DM was similar for embryos of both populations, yet embryos of population 2 were smaller than those of population 1 at 18 and 24 DPA (Fig. 4).

Endogenous hormones

Changes in ABA content during grain development were more pronounced in certain tissues and grain fractions (Fig. 5 a). At anthesis, kernel ABA content in both populations was high, but decreased by 3 DPA. Though the grain fractions of the two populations are not comparable, both populations exhibited a slight rise followed by a decrease in overall ABA between 6 and 12 DPA. This decrease continued throughout grain development. Differences in ABA content between the liquid supernatant fraction and the solid pellet fraction (population 2) were minor between 0 and 6 DPA (rapid cell division and embryo formation). Both declined in parallel through 6 DPA. Thereafter, the ABA content in the grain liquid fraction increased sharply with only a moderate decline at 24 DPA. In contrast, the ABA content in the grain pellet fluctuated little and tended to decline. Embryonic tissues from both populations contained more ABA than the surrounding kernel tissues (Fig. 5 a). From 12 to 25 DPA, the ABA content in embryos decreased slightly in population 1, but remained constant in population 2.

Fluctuations in IAA content were also tissue or fraction-dependant (Fig. 5 a). Prior to 9 DPA, the kernel IAA content was low. By 12 DPA, the kernel IAA levels had increased in all

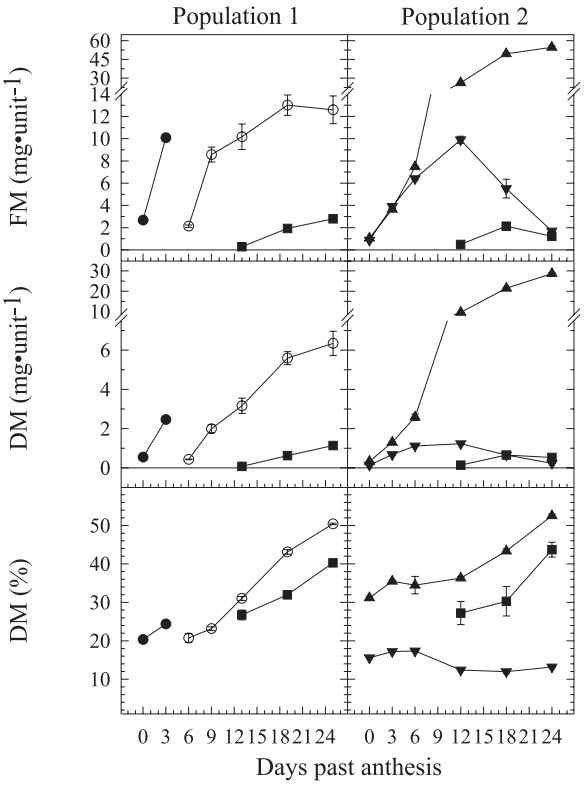

Figure 4. Mean (± SDEV) FM, DM, and percentage DM of kernel tissues from anthesis through the hard-dough stage: Population 1: whole kernels, embryos, endosperm and embryos. Population 2: embryos, centrifugal pellet from whole kernels, supernatant from whole kernels.

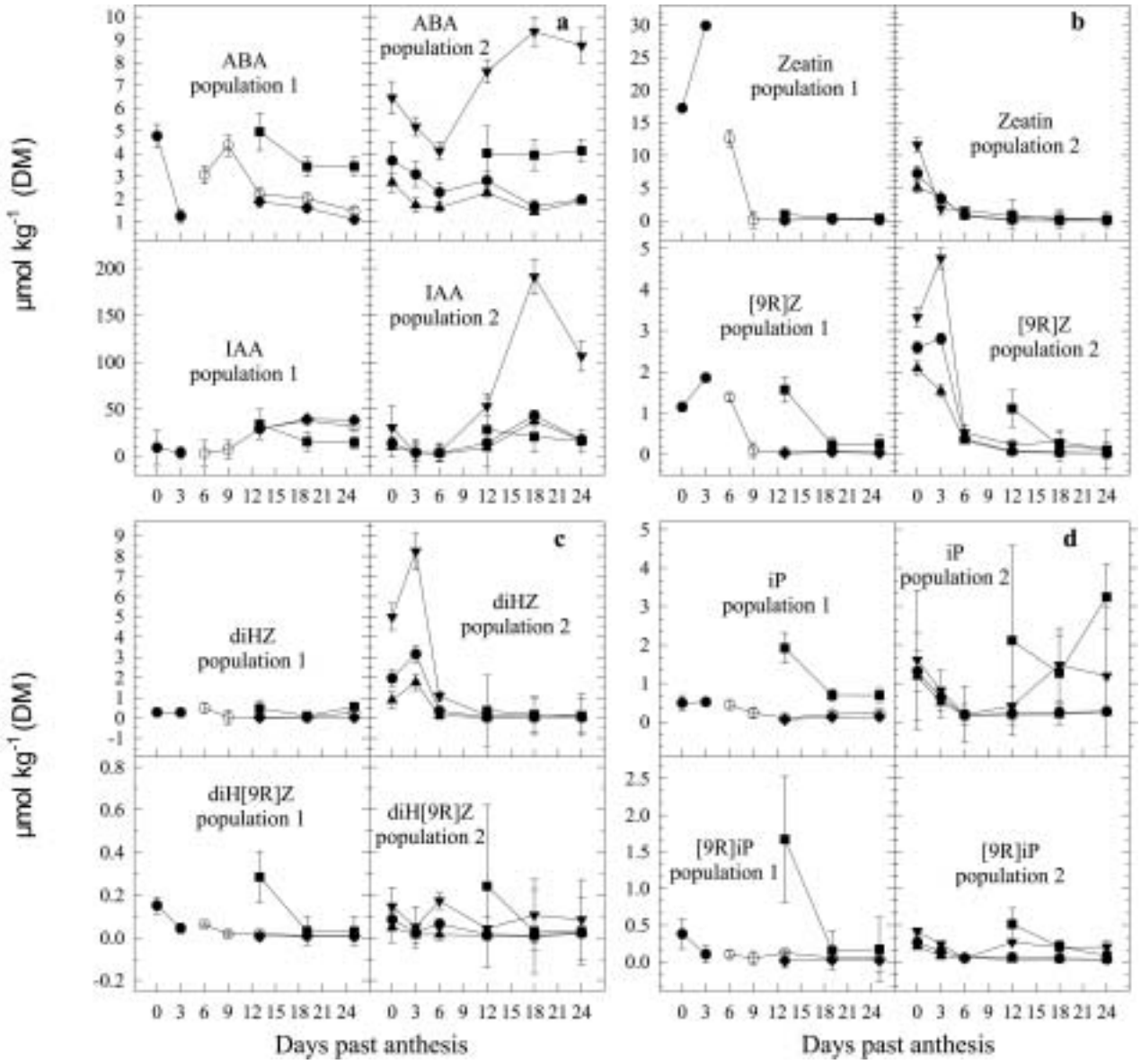

Figure 5. Mean hormone levels (± SE) of kernel tissues from anthesis to the hard-dough stage. Population 1: whole kernels, embryos, endosperm and embryos, endosperm. Populations 2: whole kernels, embryos, centrifugal pellet from whole kernels; supernatant from whole kernels.

tissues/fractions, and the IAA level in embryos was as high as or higher than in surrounding kernel tissues. These increases corresponded with rapid increases in grain size (Fig. 4). In contrast to ABA content, the IAA level in maturing embryos was significantly lower than in the surrounding kernel tissues or fractions. The IAA level in the kernel liquid fraction (population 2) rose from 3.74 µmol kg^{-1} DM at 6 DPA to 190 µmol kg^{-1} DM at 18 DPA. In both populations, IAA content for kernel tissues and fractions was highest at 18 DPA (from 20-fold to several hundred fold higher than other hormones) but declined by 25 DPA.

Distinct stage-specific cytokinin : auxin ratios were observed (Fig. 5). From 0 to 6 DPA, cytokinin content was very high and auxin content was very low. In all tissue fractions except embryos, cytokinin content, primarily Z and [9R]Z (Fig. 5 b), declined to very low levels after 6 DPA and auxin content increased.

In population 1, average levels of diHZ, diH[9R]Z (Fig. 5 c) and [9R]iP (Fig. 5 d) in non-embryonic tissues were low throughout the measurement period. In the non-embryonic tissues of population 2, diHZ exhibited similar levels and profiles to the Z cytokinins while the diH[9R]Z levels remained very low. Total iP and [9R]iP levels were low between 0 and 6 DPA but, in contrast to other cytokinins, tended to increase slightly between 13 and 25 DPA. In both populations, average iP levels were similar and exceeded levels of [9R]iP. However,

in population 2, iP content in tissues or grain fractions did not differ among collection dates (Fig. 5 d).

In population 2, differences in cytokinin content between kernel tissues and the liquid fraction depended on the particular cytokinin. From 0 to 6 DPA, cytokinin content was generally higher in the liquid fractions than in kernel tissues, but these values were similar after 6 DPA (Fig. 5 b, c, d). At 12 DPA, levels of [9R]Z, diH[9R]Z, iP and [9R]iP were higher in embryos than in kernel tissues or the kernel liquid fraction. With the exception of iP in population 1, cytokinin levels in embryonic tissues were no different than those observed in the surrounding non-embryonic tissues.

Discussion

Hormone quantification from small samples

Extraction, purification (HPLC) and quantification procedures have been optimized for ABA (Walker-Simmons 1987), IAA (Chen et al. 1988, Cohen et al. 1987), and cytokinins (MacDonald and Morris 1985). Optimizing HPLC procedures for separating the eight hormones analyzed herein was difficult because Z, [9R]Z, diHZ, and diH[9R]Z are polar, iP and [9R]iP are nonpolar, ABA tends to be nonpolar but the polarity changes with pH, and IAA is nonpolar under acidic conditions but becomes increasingly polar as the pH approaches and exceeds its pK_a of 4.5. Our procedures represent a compromise between the best extraction and fractionation procedures for each hormone and were preferable to subdividing an already limited sample. Since division was not necessary, hormone recovery percentages from original samples were increased two to three-fold. Prior to our development of this system, the cytokinin and m-IAA MAbs had not previously been used in a noncompetitive indirect ELISA configuration, but their performance, like that of the ABA MAb (Walker-Simmons 1987), was not adversely affected by the system.

Early embryony (0 to 7 DPA)

The high cytokinin levels in ovules observed during early embryony (0 to 7 DPA; Fig. 5 b, c, d) are consistent with those observed by Banowetz et al. (1999 a, b), and they probably enhance sink strength by initiating rapid cell division. During this stage, the mitotic indices of embryos (Quatrano 1987) and endosperm (Gao et al. 1992) are maximal, and solutes from the apoplast are rapidly and actively absorbed by dividing cells. This process lowers the osmotic potential of the cytoplasm, which causes water to move from the endosperm apoplast down water potenial gradients into the dividing and expanding cells (Nonami and Boyer 1987, Carman 1995). In contrast, solute movement in the phloem occurs down pressure potential and solute concentration gradients, both of which are driven by *i*) the loading of osmotically-active solutes into the phloem at sources, *ii*) the unloading and incorporation of these solutes into osmotically-inactive macromolecules at sinks, *iii*) isolation of solutes in vacuoles via active transport across tonoplasts, and *iv*) the yielding of cell membranes and walls (cell volume expansion) during cell division and differentiation (Boyer 1985, Nonami and Boyer 1987). However, cytokinin levels are also high in wheat florets in which grain fill does not occur (Lee et al. 1988). In such cases, it is assumed embryo and endosperm cells have not formed or the floret is abnormal and incapable of seed fill.

During histodifferentiation, the young embryo is not determined, and normal development is readily diverted to callus, root, shoot or somatic embryo formation by abnormal nutritional or hormonal conditions (Carman 1990, Hess and Carman 1998). The high levels of cytokinin and low levels of auxin observed in the present study during the early embryony phase (Fig. 5) are probably important to the normal sequence of embryo development. Too much cytokinin during this phase causes cotyledons to form precociously (Naylor 1984). Cytokinin levels are high during embryo histodifferentia tionin many species (Carman 1989), and the production of unique mRNAs induced by exogenous cytokinins is well documented (Mok et al. 2001). Hence, some cytokinin-induced mRNAs may commit specific regions of young embryos to form meristems or other structures.

Other hormones also appear to be involved in embryo histodifferentiation. For example, embryo development in the absence of IAA is abnormal (Naylor 1984), and this may be due to an inability to establish embryo polarity (Picciarelli et al. 2001). In contrast, ABA appears to have little effect on early embryo development. Maize and wheat embryos develop normally in ABA-deficient kernels and become dormant if exposed to ABA after embryo differentiation has occurred (Hole et al. 1989, Rasmussen et al. 1997, Suzuki et al. 2000).

Rapid growth phase (7 to 14 DPA)

IAA content in endosperm tissues increased as kernels entered the rapid growth phase (Fig. 5 a). Our IAA profiles agree with those of Bangerth et al. (1985), and similar patterns were observed for bean (Picciarelli et al. 2001). Cytokinin content in tissues surrounding the embryo declined to low levels by 7 DPA, which is similar to that observed for maize (Lur and Setter 1993), but embryo cytokinin levels tended to remain high through 12 DPA (Fig. 5 b, c, d). Increased IAA levels in differentiating tissues could enhance the sink capacity of the grain by affecting components of grain growth such as cell enlargement and nutrient accumulation. In contrast, the elevated IAA content at this stage may be coincidental (Bangerth et al. 1985).

The rapid increase in IAA content during the rapid growth phase greatly altered the auxin : cytokinin ratios in embryos and maternal tissues. These ratios are further affected by the temperature in which wheat plants are grown. Wheat plants grown in cool temperatures have higher kernel cytokinin lev-

els shortly after anthesis (Banowetz et al. 1999 a, b), a delayed onset of kernel IAA and ABA accumulation, and an increased frequency of somatic embryo formation from immature embryos (Hess and Carman 1998).

Soft-dought stage (14 to 21 DPA)

Levels of ABA and IAA increased rapidly and reached their highest points during the soft-dough stage. In contrast, cytokinin content remained low (Fig. 5). By this stage, all kernel tissues have differentiated and are committed to specific developmental pathways. However, dormancy induction (Hole et al. 1989) and maintenance (Morris et al. 1991) appear to occur during this stage. Evidence from both maize (Hole et al. 1989) and wheat (Walker-Simmons and Sesing 1990, Rasmussen et al. 1997, Suzuki et al. 2000) indicate that ABA is essential for inducing embryo dormancy at the soft-dough stage. Wheat grains that develop in ovules in which ABA synthesis is inhibited during the soft-dough stage lack post-desiccation dormancy (Rasmussen et al. 1997). Elevated levels of ABA in kernels during grain maturation are associated with high levels of dormancy in wheat (Walker-Simmons and Sesing 1990) as well as high levels of embryo storage proteins and the formation of specific mRNAs (Quatrano 1987).

Hard-dough stage (21 to 31 DPA)

Embryogenesis is nearly completed by the hard-dough stage, and maintenance of embryo dormancy during this stage appears to be an active process involving ABA-responsive mRNAs (Morris et al. 1991). Our data confirm the presence of ABA during this phase (Fig. 5 a). Furthermore, levels of iP and [9R]iP increased slightly in kernel tissues during this phase (Fig. 5 d), and similar increases were observed in oilseed rape pods (deáBouille et al. 1989). [9R]iP is a precursor of [9R]Z (Horgan 1986), and deáBouille et al. (1989) suggested that pools of iP and [9R]iP accumulate when not required as precursors for the respective Z forms. This may explain the low levels of iP and [9R]iP observed in relation to high levels of the Z forms during early embryony (Fig. 5 b, d), since rapid increases in Z form cytokinins would deplete the iP and [9R]iP pools.

Implications for *in vitro* culture systems

Dynamic changes in hormone levels occurred in the ovule as kernels progressed through each stage of development, and these changes probably play important roles in normalizing embryony. Some developmental events may rely on a single hormone, while others may require multiple hormones as well as other internal and external factors. The data presented herein may be useful in developing dynamic, multiple factor culture procedures that normalize zygotic and somatic embryony *in vitro* (Hess and Carman 1993 1998, Carman 1995, Carman et al. 1996).

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